A STUDY OF THE REACTION KINETICS OF A CATHEPTIC ENZYME WITH A DECARBOXYLASE AS INDICATOR*

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Cathepsin activities have frequently been reported in terms of first order reaction constants (1, 2). The assumption has been made that such a procedure is justifiable at the low substrate concentrations usually employed (3). Using a manometric method (4) well adapted to the study of enzyme kinetics, we have found that hydrolysis by swine kidney pepsinase follows a biphasic curve, at substrate concentrations even lower than those usually employed by other workers. The purposes of this paper are to present the evidence for these statements and to analyze further the reactions involved.

EXPERIMENTAL

Preparation of the enzymes and the experimental methods were as previously described (4).

Fig. 1 represents an experiment in which the rates of splitting of two concentrations of carbobenzoxy-L-glutamyl-L-tyrosine were measured by addition of decarboxylase. Fig. 1 illustrates several characteristics of the system. (*a*) An initial delay occurs at the beginning of the reaction, before the maximum velocity is attained (*cf.* also Fig. 3). This delay appears to be due in part to the time required for the tyrosine concentration to rise to the constant value maintained during the steady state. The delay is minimized by use of a large excess of decarboxylase. (*b*) In this range of concentrations, the rate is independent of the substrate concentration, and the reaction may be said to follow zero order kinetics. (*c*) As the reaction proceeds, the rate falls. This fall cannot be due directly to substrate depletion. In Curve A of Fig. 1, the substrate concentration after 220 minutes remains greater than the initial concentration for Curve B, and yet the initial rates are almost identical.

In an attempt to explain the fall in rate, flasks were incubated for varying

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lengths of time, prior to tipping. Fig. 2 shows that no appreciable inactivation of the cathepsin occurred.

Evidence was found, however, that accumulation of one of the split-products causes a decrease in the rate. Fig. 3 illustrates the inhibiting effect of the addition of carbobenzoxy-L-glutamic acid initially. Addition of the other end-product, tyramine, did not alter the rate. Agreement between the decarboxylase and amino nitrogen methods (4) suggests that accumulation of tyrosine has little effect. This observation also provides evidence against the unlikely possibility that the slowing of the reaction is due to a mass law effect, with reversal of the hydrolysis.

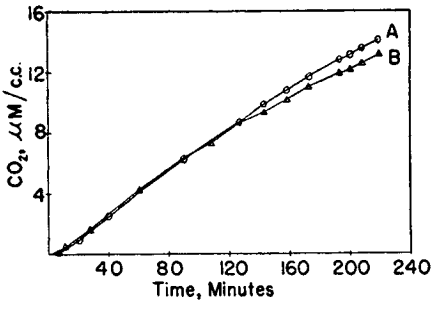

FIG. 1

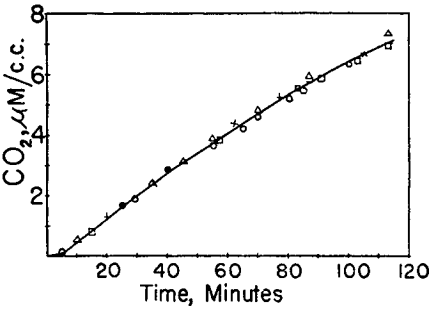

FIG. 2

FIG. 1. Course of the reaction at high concentrations of substrate. Swine kidney cathepsin, 0.1 cc. (Curve A), 0.1 cc. (Curve B); decarboxylase, 0.6 cc. (Curve A), 0.6 cc. (Curve B); carbobenzoxy-L-glutamyl-L-tyrosine, final concentration in test solution, micromoles per cc., 58 (Curve A), 29 (Curve B). Total volume in each vessel 1.3 cc.; pH 5.65; temperature 25.0°.

FIG. 2. Effect on catheptic activity of incubation at 25°. Composition of solution in each vessel: decarboxylase 0.7 cc.; carbobenzoxy-L-glutamyl-L-tyrosine, 0.055 M, 0.3 cc.; swine kidney cathepsin 0.1 cc. The vessels from which the five sets of points were obtained were incubated for varying lengths of time, as follows: ○ experiment begun immediately, △ incubated for 30 minutes before the substrate was tipped into the enzyme solution, □ incubated for 60 minutes, × incubated for 150 minutes, ● incubated for 260 minutes.

Fig. 4 shows the course of the reaction for four different concentrations of substrate. In Fig. 5 the equilibrium constants for formation of the enzyme-substrate and enzyme-inhibitor complexes have been evaluated by the graphical method of Lineweaver and Burk (5). The constancy of the ordinate intercept suggests that the inhibition is actually competitive.

Interpretation

The following scheme seems to describe fairly well the reactions which occur in this system.

$$\text{Substrate} + \text{cathepsin} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{cathepsin-substrate complex} \xrightarrow{k_3}$$

$$\text{carbobenzoxy-L-glutamic acid} + \text{L-tyrosine} + \text{cathepsin}$$

$$\text{L-Tyrosine} \xrightarrow[k_4]{\text{decarboxylase}} \text{tyramine} + CO_2$$

$$\text{Carbobenzoxy-L-glutamic acid} + \text{cathepsin} \underset{k_6}{\overset{k_5}{\rightleftarrows}}$$

$$\text{carbobenzoxy-L-glutamic acid-cathepsin complex}$$

In the discussion of these reactions, the following symbols will be used.

S = concentration of carbobenzoxy-L-glutamyl-L-tyrosine in micromoles per cc. at time t
S_0 = initial concentration of carbobenzoxy-L-glutamyl-L-tyrosine
t = time in minutes
W = amount of carbon dioxide evolved per cc. of test solution, in micromoles, up to time t
I = concentration of carbobenzoxy-L-glutamic acid in micromoles per cc. at time t
Z = tyrosine concentration in micromoles per cc. at time t
E = cathepsin concentration in arbitrary units at time t
E_0 = initial cathepsin concentration
v = velocity of hydrolysis in micromoles per minute per cc. of test solution
V = k_3E_0, the velocity of hydrolysis when the enzyme is saturated
$K_s = \dfrac{k_2 + k_3}{k_1}$, $K_i = \dfrac{k_6}{k_5}$

In the development of this method for practical use, relatively large concentrations of decarboxylase have been used to make the cathepsin activity the dominant factor determining the over-all velocity. The tyrosine concentration is always extremely low. Under these conditions, it seems justifiable to assume that the decarboxylation follows first order kinetics. The differential equation for the final reaction may then be expressed as follows:

$$\frac{dW}{dt} = k_4 Z \tag{1}$$

If we neglect the small amount of substrate tied up in intermediate complexes, the initial substrate concentration should equal the sum of the substrate concentration at time t, the tyrosine concentration, and the amount of carbon dioxide evolved.

$$S_0 = S + Z + W \tag{2}$$

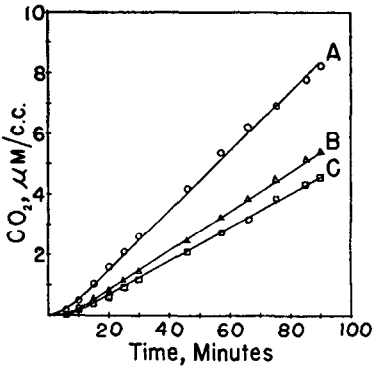

FIG. 3

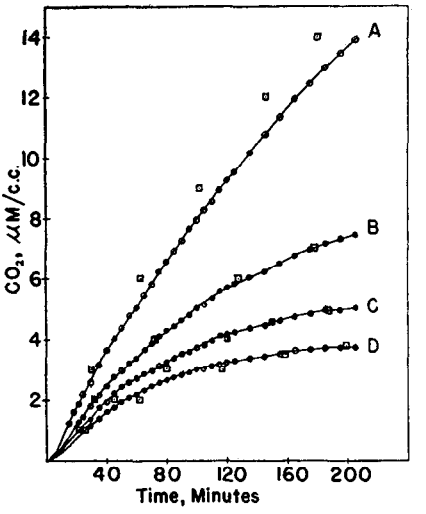

FIG. 4

FIG. 3. Effect of the addition of carbobenzoxy-L-glutamic acid on the activity of cathepsin. The compositions of the solutions in the Warburg vessels were as follows: swine kidney cathepsin 0.1 cc.; carbobenzoxy-L-glutamyl-L-tyrosine, 0.125 M, 0.3 cc.; decarboxylase 0.4 cc.; carbobenzoxy-L-glutamic acid, 0.125 M, 0 cc. (Curve A), 0.15 cc. (Curve B), 0.3 cc. (Curve C). All volumes were made up to 1.1 cc.; pH 5.6; temperature 25.0 °.

FIG. 4. Effect of varying substrate concentrations. Substrate concentrations, micromoles per cc., 30.3 (Curve A), 10.8 (Curve B), 6.5 (Curve C), 4.7 (Curve D). Other constituents in each flask: swine kidney cathepsin 0.1 cc.; decarboxylase 0.6 cc. Total volume in each flask 1.3 cc.; pH 5.4; temperature 25.0°. Theoretical points for each concentration are represented by □, and were calculated by means of equation (12). The values for V, K_s, and K_i were calculated as described under Fig. 5.

Solving for Z and substituting in equation (1), we obtain

$$\frac{dW}{dt} = k_4 S_0 - k_4 S - k_4 W \tag{3}$$

A more convenient form for integration may be obtained by multiplying equation (3) by dt, adding $k_4 W dt$ to both sides, and multiplying by the factor $e^{k_4 t}$ to convert the left hand member to an exact differential.

$$\int (e^{k_4 t}\, dW + k_4 W e^{k_4 t}\, dt) = \int k_4 S_0 e^{k_4 t}\, dt - \int k_4 S e^{k_4 t}\, dt \tag{4}$$

$$W = S_0 - \frac{\int k_4 S e^{k_4 t}\, dt}{e^{k_4 t}} \tag{5}$$

Equation (5) furnishes a means of calculating the amount of carbon dioxide evolved, provided we can find S, the substrate concentration, in terms of t and then evaluate the integral. Under certain conditions, these operations are possible. First, let us consider the simplest case, when the

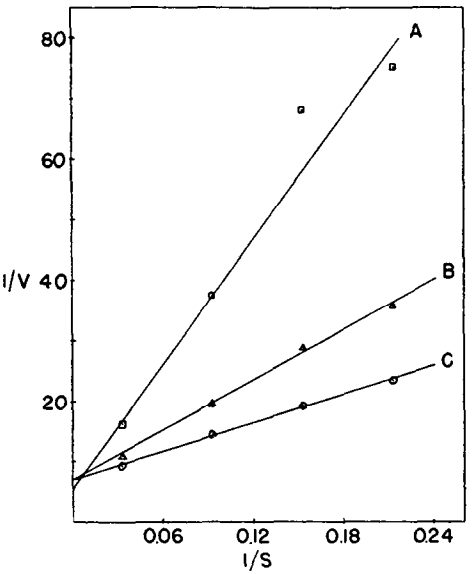

FIG. 5. Evaluation of V, K_s, and K_i. v is expressed in micromoles per cc. per minute and S in micromoles per cc. Concentration of carbobenzoxy-L-glutamyl-L-tyrosine, micromoles per cc., 30.3, 10.8, 6.5, 4.7. Concentration of carbobenzoxy-L-glutamic acid, micromoles per cc., 30.3, 30.3, 30.3, 30.3 (Curve A); 7.58, 7.58, 7.58, 7.58 (Curve B); 0 for each (Curve C). Other conditions were the same as for Fig. 4. $1/V$ is represented by the ordinate intercept. The value 6.5 was used in the calculations, giving 0.154 micromoles per cc. per minute for V; K_s/V is represented by the slope of Curve C; K_s was calculated to equal 12.6 micromoles per cc., or 0.0126 mole per liter. Sufficient data have not been collected to permit calculation of a statistically significant Michaelis constant. The one arrived at in this experiment happens to be the highest that has been obtained. Experiments with different cathepsin preparations have led to values as low as 0.0041 mole per liter. K_i was calculated from the following relation, $K_s + K_s I/K_i = V$ times the slope of the line. Curve A gives a value of 0.0094 and Curve B 0.0110 mole per liter. For the theoretical points on Fig. 4, the value 0.01 was used.

substrate concentration is so large that the rate of hydrolysis is independent of it and is determined entirely by the cathepsin concentration. Referring to the system of velocity constants originally defined, we may write

$$-\frac{dS}{dt} = k_3 E_0 \tag{6}$$

Integrating and evaluating the constant of integration, we obtain

$$S = S_0 - k_3 E_0 t \tag{7}$$

Substituting this value for S in equation (5), integrating, and evaluating the constant of integration, we find

$$W = k_3 E_0 t - \frac{k_3 E_0}{k_4} (1 - e^{-k_4 t}) \tag{8}$$

Next, let us consider the situation in which the initial substrate concentration is so low that the hydrolysis is first order. Under these conditions, the substrate concentration will fall along a "die away" curve.

$$S = S_0 e^{-k_1 t} \tag{9}$$

Substituting this value in equation (5), integrating, evaluating the constant of integration, and simplifying, we obtain

$$W = S_0 \left[1 - \frac{1}{k_4 - k_1} (k_4 e^{-k_1 t} - k_1 e^{-k_4 t}) \right] \tag{10}$$

Equation (10) is identical with that given by Daniels (6) for consecutive monomolecular reactions.

To describe the conditions actually existing at various concentrations of cathepsin and substrate, we must include both zero and first order terms. Inhibition by one of the split-products must also be considered. As direct an approach as any to this problem begins with an equation given by Lineweaver and Burk (5) for velocity in the presence of a competitive inhibitor.

$$\frac{1}{v} = \frac{1}{V} \left[K_s + \frac{K_s I}{K_i} \right] \frac{1}{S} + \frac{1}{V} \tag{11}$$

A detailed derivation of equation (11) is presented by Wilson (7).

In the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine, the concentration of inhibitor, I, is not constant, but is equal to the amount of substrate decomposed, $S_0 - S$. If we substitute this value for I in equation (11), substitute $-dS/dt$ for v, integrate, and evaluate the constant of integration,

$$t = \frac{K_i K_s + K_s S_0}{V K_i} \ln \frac{S_0}{S} + \frac{K_i - K_s}{V K_i} (S_0 - S) \tag{12}$$

Michaelis and Menten (8), studying the splitting of sucrose by invertase, found that both split-products were inhibitory. They derived an equation which reduces to equation (12) if inhibition by one split-product is neglected.

Equation (12) provides a relation between the time and the amount of substrate hydrolyzed, throughout the biphasic reaction. In the presence

of a large excess of decarboxylase, the amount of carbon dioxide produced may be considered to be equivalent to the amount of substrate decomposed, and the theoretical course of the reaction may be plotted. Fig. 4 shows a comparison of the observed and calculated curves over a limited range of substrate concentrations.

DISCUSSION

It is interesting to notice that equation (12) becomes formally identical with an integrated equation derived by Van Slyke and Cullen (9), if $1/K_i = 0$, implying no inhibition. In the derivation by Van Slyke and Cullen, however, K_s equals k_3/k_1, k_2 is neglected, and K_s varies with k_3. In the case of urease, this situation seems to hold. Chance (10), studying peroxidase, was able to measure the three constants directly, and also found k_2 to be very small in comparison with k_3. Michaelis and Menten (8), on the other hand, assumed that k_3 was negligible in comparison with k_1 and k_2. Lineweaver, Burk, and Deming (11), studying nitrogenase in *Azotobacter*, deduced evidence in support of the original assumption by Michaelis and Menten. Briggs and Haldane (12) pointed out the fact that K_s actually represents $(k_2 + k_3)/k_1$.

Chance (10) obtained particular solutions of the differential equations of the Michaelis theory by means of the differential analyzer. His theoretical curves differ slightly from those plotted from the equations discussed above in that they include a very brief increased rate at the beginning, while the enzyme-substrate complex is building up to its maximum value. The methods of obtaining general solutions of the differential equations employed above, and by Michaelis and Menten, Van Slyke and Cullen, and Briggs and Haldane, succeed only because the assumption is made that the rate of change in concentration of the enzyme-substrate complex is negligible in comparison with the rate of disappearance of substrate. Obviously this assumption is not valid at the beginning of the reaction.

The behavior of the terms involving K_i in equation (12) is interesting at low values of $S_0 - S$. As $S_0 - S$ approaches 0, each of these terms also approaches 0. By expansion of the logarithmic term in Maclaurin's series, however, it may be shown that the sum of these terms constitutes a second order infinitesimal with respect to $S_0 - S$. This relationship is another way of stating that early in the reaction inhibitory effects due to the split-products are negligible.

SUMMARY

1. The kinetics of the splitting of carbobenzoxy-L-glutamyl-L-tyrosine by swine kidney pepsinase have been studied, with the aid of a tyrosine decarboxylase.

2. The hydrolysis has been shown to follow a biphasic curve, with zero order kinetics early in its course.

3. One of the split-products, carbobenzoxy-L-glutamic acid, has been shown to inhibit the catheptic activity.

4. Over a limited range of substrate concentrations, the experimental curves have been shown to agree fairly well with theoretical curves based on the theory of formation of an enzyme-substrate complex.

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